

# Phospholipase C activation and $\text{Ca}^{2+}$ mobilization by cloned human somatostatin receptor subtypes 1–5, in transfected COS-7 cells

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## Abstract

We transfected the COS-7 cells with cDNAs encoding different human somatostatin receptor (hSSTR) subtypes, and found that hSSTR subtypes mediate not only the inhibition of forskolin-induced cAMP accumulation but also the stimulation of phospholipase C (PLC) and  $\text{Ca}^{2+}$  mobilization. Activation of PLC by 1  $\mu\text{M}$  somatostatin (SRIF) was in the order of: hSSTR5 > hSSTR2 > hSSTR3 > hSSTR4 >> hSSTR1. Pertussis toxin (PTX) treatment completely or partially reversed the PLC activation. 1 nM SRIF was equally effective for adenylate cyclase (AC) inhibition in a PTX-sensitive manner, in all the cells expressing different hSSTRs, except for hSSTR1. Nevertheless, SRIF stimulated AC even in the presence of forskolin at higher doses of SRIF in PTX-treated hSSTR5-expressing cells. We conclude that the cloned hSSTRs differentially couple to PTX-sensitive and -insensitive G-proteins to modulate PLC,  $\text{Ca}^{2+}$  mobilization and AC.

**Key words:** Somatostatin receptor subtypes; Phospholipase C; Adenylate cyclase; G-proteins; COS-7 cell

## 1. Introduction

Somatostatin (SRIF), a tetradecapeptide, exerts a variety of physiological effects in different organs [1]. For example, in central nervous system SRIF acts as a neurotransmitter [2] and neuromodulator, both in an excitatory and inhibitory manner [3,4]. In pituitary and pancreas it is a potent inhibitory regulator of secretory processes [1]. Recent studies have demonstrated the antiproliferative effects of SRIF in tumor cells derived from different tissues including pituitary, breast and prostate [5]. Most, but not all, SRIF actions are sensitive to pertussis toxin (PTX), e.g. PTX treatment diminishes SRIF inhibition of the secretion of hormones and enzymes [1], but SRIF-induced  $\text{Na}^+/\text{H}^+$  exchange and cell growth inhibition [7] are not sensitive to PTX [6]. These findings suggest the coupling of the SRIF receptor to a variety of effector systems through PTX-sensitive and -insensitive G-proteins, to regulate multiple cellular processes.

Until now, five subtypes of hSSTR (hSSTR1, hSSTR2, hSSTR3, hSSTR4, and hSSTR5) have been cloned [8–14]. Among them hSSTR2, hSSTR3, and hSSTR4 but not hSSTR5 have been reported to couple to an inhibitory adenylate cyclase system through PTX-sensitive G-proteins [14,15]. Thus, in spite of a variety of

SRIF functions, on signal transduction pathways coupled to the cloned SRIF receptors only the adenylate cyclase system has been studied. Hence, it is important to investigate the other effector systems such as phospholipase C (PLC) and  $\text{Ca}^{2+}$  mobilization. Recently, we have reported [15] the activation of the PLC- $\text{Ca}^{2+}$  system by hSSTR1 and hSSTR2. In this study we characterize all the other receptor subtypes regarding PLC activation and  $\text{Ca}^{2+}$  mobilization along with adenylate cyclase modulation. In order to better compare some responses we used hSSTR1- and hSSTR2-transfected cells.

## 2. Materials and methods

### 2.1. Materials

SRIF and forskolin were purchased from Sigma; [ $^{125}\text{I}$ ]Tyr<sup>1</sup>-SRIF (2,200 Ci/mmol) from DuPont-New England Nuclear. PTX was kindly provided by Dr. M. Ui (Tokyo Univ.), and the cAMP assay kit was from Yamasa Shoyu Co. (Chiba, Japan). The sources of all other reagents were the same as described in [15,16].

### 2.2. Cell culture and DNA transfection

COS-7 cells were cultured and harvested as described in [15]. All expression vectors were constructed as described in [9,13,15] and transfected in COS-7 cells by electroporation as described in [15,16].

### 2.3. Inositolphosphate assay

The cells buffered with [ $^3\text{H}$ ]inositol were washed twice with the HEPES-buffered medium composed of 10 mM HEPES (pH 7.5), 134 mM NaCl, 4.7 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4$ , 2 mM  $\text{CaCl}_2$ , 2.5 mM  $\text{NaHCO}_3$ , 5 mM glucose, and 0.1% (w/v) bovine serum albumin (fraction V), and then incubated for 1 h at 37°C with the same medium containing 10 mM LiCl and the agents to be tested in a final volume of 0.5 ml. The reaction was terminated by aspirating the medium and adding 1.2 ml of 5% perchloric acid. [ $^3\text{H}$ ]inositol phosphates including inositol mono-, di- and tri-phosphates were separated on Dowex 1  $\times$  8 formate columns as described in [17].

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**Abbreviations:** SRIF, somatostatin; PTX, pertussis toxin; hSSTR1–5, human somatostatin receptor type 1–5; G-protein, guanine-nucleotide binding regulatory protein;  $\text{G}_i$  and  $\text{G}_s$ , inhibitory and stimulatory G-proteins;  $[\text{Ca}^{2+}]_i$ , cytosolic  $\text{Ca}^{2+}$  concentration.

#### 2.4. $[Ca^{2+}]_i$ measurements

Cytosolic  $Ca^{2+}$  was estimated by monitoring changes in the fluorescence of Fura-2 loaded cells as described in [15,16].

#### 2.5. Radioimmunoassay of cAMP content

The cells were washed twice with the HEPES-buffered medium, and incubated for 10 min at 37°C, with the agents to be tested in the presence of 100  $\mu$ M Ro 20-1724 and 50  $\mu$ M forskolin in all cAMP experiments, unless otherwise stated. The reaction was terminated by adding 100  $\mu$ l of 1 N HCl. Measurement of cAMP content was performed as described in [16].

#### 2.6. $[^{125}I]$ Tyr<sup>1</sup>-SRIF binding

SRIF binding experiments were performed by the methods as described in [8,15] with slight modifications. Briefly cells were washed with the HEPES-buffered medium, and then incubated in the same medium with  $[^{125}I]$ Tyr<sup>1</sup>-SRIF (ca. 40,000 cpm) in the presence of 1.2 nM SRIF for 30 min at 25°C. The cells were washed twice with the ice-cold HEPES-buffered medium and membrane bound  $[^{125}I]$ SRIF was extracted with 5% trichloroacetic acid (1 ml). Radioactivity in the extract was measured by a  $\gamma$ -counter. Specific binding was defined as the radioactivity displaced by the addition of 1  $\mu$ M unlabeled SRIF.

#### 2.7. Data presentations

All experiments were performed in duplicate or triplicate. The results of multiple observations are presented as the mean of results from more than three different batches of the cells, unless otherwise stated.

### 3. Results

Fig. 1 shows the typical patterns of 100 nM SRIF-induced increase in  $[Ca^{2+}]_i$  in the cells transfected with different SRIF receptor cDNAs. SRIF did not induce any  $[Ca^{2+}]_i$  increase in the cells transfected with plasmid vector alone even at 1  $\mu$ M concentration, but in all the cells transfected with three hSSTR cDNA subtypes, a significant increase in  $[Ca^{2+}]_i$  was observed. Such increase in  $[Ca^{2+}]_i$  was found even at low extracellular  $Ca^{2+}$  conditions where an excess amount of EGTA was added before application of the agonist (data not shown). In hSSTR5-expressing cells, the  $[Ca^{2+}]_i$  level reached about 1.5 times the basal value, and was higher than those in hSSTR3- and hSSTR4-expressing cells. The net increases in  $[Ca^{2+}]_i$  by 100 nM SRIF in hSSTR5-, hSSTR3-,

and hSSTR4-expressing cells were  $69.75 \pm 6.8$ ,  $38.5 \pm 2.5$  and  $22.5 \pm 0.5$  nM, respectively.

In order to further characterize the hSSTR-mediated  $[Ca^{2+}]_i$  increase, we next analyzed PLC activation mediated by the aforementioned receptor subtypes in comparison with hSSTR1 and hSSTR2. Fig. 2A shows the inositol phosphate production by SRIF. In agreement with the  $[Ca^{2+}]_i$  increases, the inositol phosphate response to 1  $\mu$ M SRIF in hSSTR5-expressing cells was higher (12 times the basal activity) than those in hSSTR3 and hSSTR4-expressing cells (2.7 and 1.7 times, respectively). Consistent with previous results [15], the inositol phosphate response in hSSTR2-expressing cells was much higher (10 times the basal activity) than that in hSSTR1-expressing cells (1.3 times). Thus, the order of potency for SRIF-induced inositol phosphate production was hSSTR5 > hSSTR2 > hSSTR3 > hSSTR4 >> hSSTR1.

As shown in Fig. 2B, PTX treatment of the cells inhibited PLC activities mediated by all the hSSTR subtypes. The inhibition was more obvious at the lower doses of SRIF, although in the case of hSSTR4-expressing cells, more than 90% inhibition occurred within the whole range of SRIF concentrations tested. The inhibition was 60%, 80% and 90% for 10 nM, and 35%, 55% and 70% for 1  $\mu$ M in hSSTR5-, hSSTR2- and hSSTR3-expressing cells, respectively (Fig. 2B).

To rule out the possibility that the quantitative differences in effects of the expressed hSSTR subtypes might be due to differential expression of receptor proteins on the surface of transfected cells, we examined specific binding of labeled SRIF on the transfected cells. As shown in Fig. 3, at 1.2 nM  $[^{125}I]$ SRIF binding on the cells transfected with hSSTRs was in the order of: hSSTR1 > hSSTR4 > hSSTR2 > hSSTR5 = hSSTR3. These results confirm clearly the higher efficacy of hSSTR5- and hSSTR2-expressing cells, when compared to hSSTR1 and hSSTR4 in coupling to the PLC/ $Ca^{2+}$  signaling pathway.

Concurring with our previous study [15], SRIF inhib-

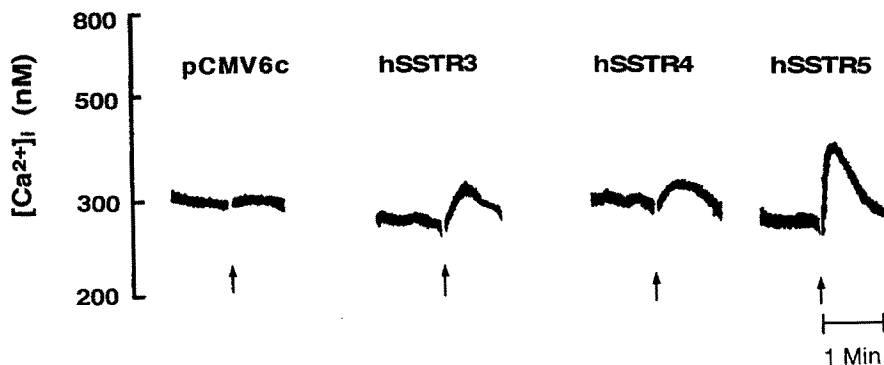


Fig. 1. Representative traces of the  $[Ca^{2+}]_i$  changes produced by SRIF (100 nM) added at the sites indicated by arrows in pCMV6c-transfected, hSSTR3-, hSSTR4-, and hSSTR5-expressing, Fura 2-loaded cells.

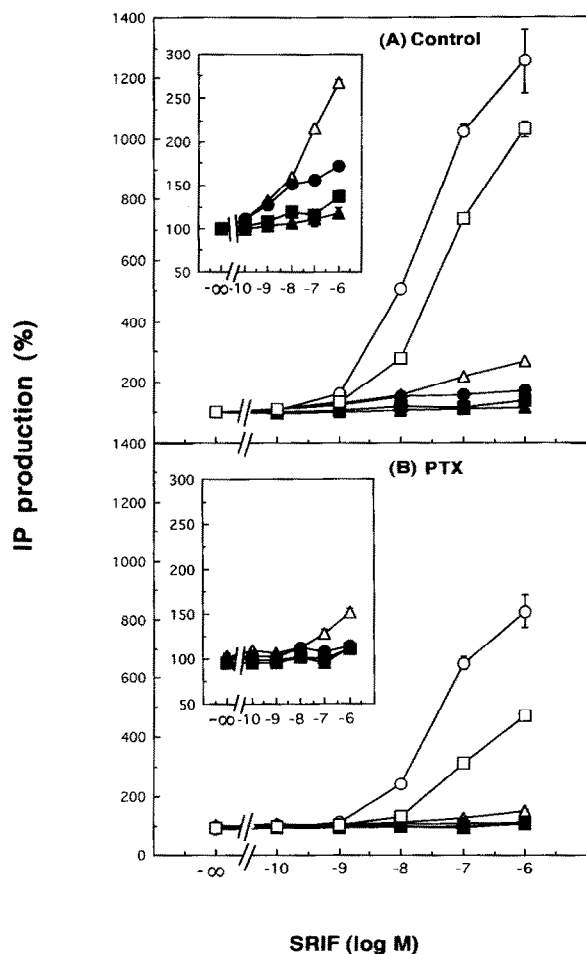


Fig. 2. Dose-dependent effect of SRIF on inositol phosphate production (including mono-, di-, and triphosphates) in pCMV6c-transfected (▲), hSSTR1 (■), hSSTR2 (□), hSSTR3 (△), hSSTR4 (●), and hSSTR5 (○)-expressing cells, untreated (A) and treated (B) with PTX (100 ng/ml, for 18 h before assays). Insets in A and B show the same activity (except hSSTR2 and hSSTR5) at an enlarged scale. The results are shown as mean  $\pm$  S.E.M. of three different experiments performed in triplicate and expressed as percentage of the control value without SRIF, taken as 100%.

ited forskolin-induced cAMP accumulation in cells transfected with plasmid vector alone, showing that the cells contain some native low affinity SRIF receptors (Fig. 4A). On the other hand, we found an efficient coupling of hSSTR3, hSSTR4, and hSSTR5 to the adenylate cyclase system. SRIF dose-dependently inhibited forskolin-induced cAMP accumulation in the cells transfected with hSSTR3, hSSTR4, and hSSTR5, even at low doses of SRIF (0.1–1 nM) (Fig. 4B,C,D). Surprisingly, however, the inhibitory effect mediated by hSSTR5 was significantly reduced with the increase in SRIF concentrations above 10 nM.

PTX treatment of the transfected cells completely abolished the inhibitory effect of SRIF on the forskolin-induced cAMP accumulation in the hSSTR3- and

hSSTR4-expressing cells as well as in the cells transfected with vector alone. Interestingly, in the cells expressing hSSTR5, in addition to the complete abolition of the inhibitory effect, there was a further increase in cAMP accumulation in response to SRIF, especially at higher doses.

#### 4. Discussion

In our COS-7 cell expression system, as we reported recently [15], hSSTR2, and to a lesser extent hSSTR1, induced activation of PLC and  $\text{Ca}^{2+}$  mobilization. We now are able to report the characterization of the other hSSTR subtypes, hSSTR3, hSSTR4, and hSSTR5, and their responses to SRIF, which are compared with those of hSSTR1 and hSSTR2. As shown in Fig. 2, maximum activation of PLC at 1  $\mu\text{M}$  SRIF was in the order of hSSTR5 > hSSTR2 > hSSTR3 > hSSTR4 >> hSSTR1, in a PTX-sensitive manner. However, the mode of inhibition caused by PTX was dependent upon the species of receptor subtype and the SRIF dose employed. Thus, all the hSSTR subtypes mediate the SRIF activation of PLC preferentially through a PTX-sensitive pathway at lower doses of SRIF, but hSSTR2, hSSTR3, and hSSTR5 also are able to do so through a PTX-insensitive pathway at higher doses. Analogically to the other seven-transmembrane type receptors coupled to PLC, the PTX-sensitive and -insensitive pathways probably involve  $\text{G}_i/\text{G}_o$  and  $\text{G}_q$  (or other members of its family), respectively [18]. Taken together, these results suggest that all the hSSTR subtypes preferentially couple to  $\text{G}_i/\text{G}_o$  at lower doses of

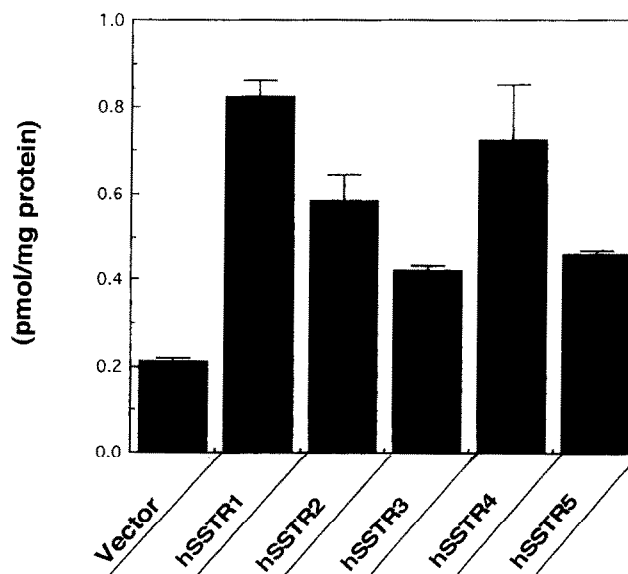


Fig. 3. Specific binding of [ $^{125}\text{I}$ ]Tyr<sup>1</sup>-SRIF to pCMV6c-transfected, hSSTR1-, hSSTR2-, hSSTR3-, hSSTR4-, and hSSTR5-expressing cells in 6-well plates (about 100  $\mu\text{g}$  cell protein) were incubated with [ $^{125}\text{I}$ ]SRIF in the presence of 1.2 nM SRIF. Results were normalized and specific binding (pmol/mg of protein) was expressed as mean  $\pm$  S.E.M. of four values from two separate experiments.

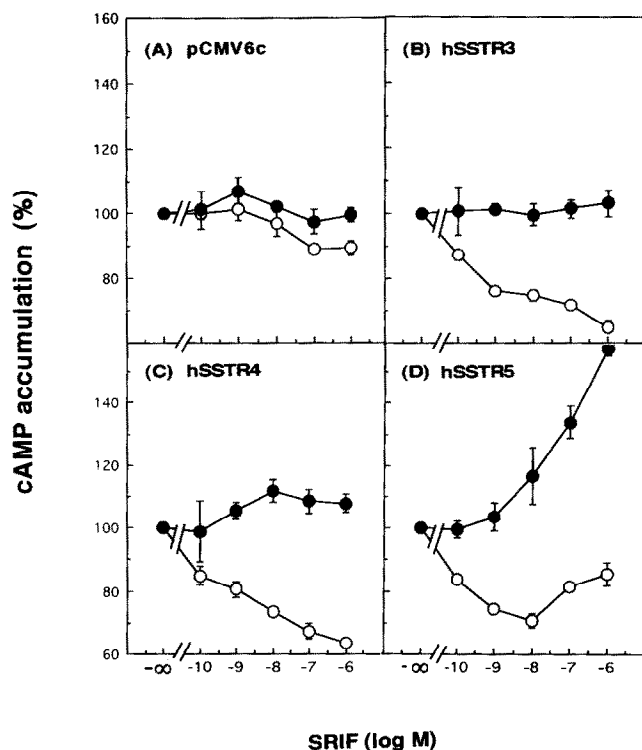


Fig. 4. Dose-dependent effect of SRIF on forskolin-induced cAMP accumulation in pCMV6c-transfected (A), hSSTR3- (B), hSSTR4- (C), and hSSTR5- (D) expressing cells, untreated (○) and treated (●) with PTX (100 ng/ml for 18 h before assay). The results are shown as mean  $\pm$  S.E.M. of three separate experiments performed in triplicate.

SRIF, but hSSTR2, hSSTR3 and hSSTR5 can also couple to  $G_q$  to activate PLC at higher doses. This conclusion is supported by the fact that the lower doses of SRIF (at 0.1–1 nM) inhibited adenylate cyclase in a PTX-sensitive manner in hSSTR3-, hSSTR4-, and hSSTR5- (Fig. 4) as well as in hSSTR2-expressing cells [15].

Raynor et al. [14] have reported that SRIF did not inhibit cAMP accumulation either in COS-1, CHO-DG44, or in CHO-K1 cells expressing hSSTR5, but in our COS-7 cell expression system, we could detect a significant inhibitory effect of SRIF. This discrepancy between our results and Raynor's might be due to the differences in the amount and species of G-proteins expressed in COS-7 cells from those in the cell types examined by them. Even in our study, however, the cAMP response pattern seems to be odd. The inhibitory effect on the forskolin-induced cAMP accumulation was appreciably reduced at higher doses of SRIF. Furthermore, in PTX-treated cells, the inhibitory effect of SRIF on forskolin-induced cAMP accumulation was completely abolished, and in contrast, the high cAMP level obtained by forskolin was further increased in response to SRIF (Fig. 4D). We also found that in the absence of forskolin, SRIF did not inhibit but stimulated cAMP accumulation in a dose dependent manner (data not shown). The po-

tency of SRIF in the reaction was similar to that in the induction of inositol phosphate response (data not shown), suggesting that hSSTR5 can link to the adenylate cyclase system as a stimulator in addition to inhibitory role at the higher doses of SRIF. We have no direct evidence showing the coupling of hSSTR5 to  $G_s$ . When the COS-7 cells were transfected with muscarinic receptor type 3, which is known to couple to a PTX-insensitive G-protein to activate PLC, but not to  $G_s$ , carbachol increased not only inositol phosphate production to a similar extent as that induced by SRIF but also cAMP accumulation, although to a lesser extent than in the case of SRIF (data not shown). Among several subtypes of adenylate cyclases, type 2, 5 and 6 have been shown to be activated by receptors coupled to PLC through protein kinase C activation and  $Ca^{2+}$  mobilization [18]. Such a mechanism might at least in part be involved in SRIF-induced cAMP accumulation in hSSTR5-expressing cells.

In a few studies, SRIF activated PLC followed by  $Ca^{2+}$  mobilization in native SRIF receptor-expressing cells such as striatal astrocytes [19] and NG 108-15 [20]. The requirement of  $Ca^{2+}$ -dependent phospholipase  $A_2$  activation also has been postulated in SRIF actions in hippocampal cells [21]. Since SRIF exerts excitatory as well as inhibitory effects on neurons [4], activation of PLC and related responses may account for the excitation of somatostatinergic neurons in central nervous system.

Our findings in the present study suggest that cloned human SRIF receptor subtypes, when transfected in COS-7 cells, not only modulate the adenylate cyclase system but also couple to PTX-sensitive and -insensitive G-proteins to stimulate PLC and  $Ca^{2+}$  mobilization, providing a new insight into the transducing cascade in various physiological events.

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